Isolation and Identification of Measles Virus in Cell Culture

Revised November 29, 2001

Centers for Disease Control and Prevention
National Center for Infectious Diseases
Division of Viral and Rickettsial Diseases
Respiratory and Enteric Viruses Branch
Measles Virus Section

Pan American Health Organization
Pan American Sanitary Bureau Regional Office of the
World Health Organization
Division of Vaccines and Immunization

Contents

I. Introduction

The availability of a sensitive cell line (B95a) for isolation of measles virus from clinical specimens and establishment of automated DNA sequencing techniques have allowed for rapid genetic characterization of a large number of wild-type strains of measles virus. This database of sequence information now makes it possible to use molecular epidemiologic techniques to identify the source of wild-type viruses and to differentiate between wild-type and vaccine strains (1-4).

As progress is made toward elimination of measles in many regions of the world, it will be critical to examine virus isolates from as many outbreaks and isolated cases as possible in order to identify the source of the virus. The World Health Organization (WHO) held a meeting in May 1998 to standardize the protocols for the genetic characterization of wild-type measles viruses and to establish a consistent system for describing the genotypes (5). Collection of measles specimens will help to determine which outbreaks may be related and to monitor patterns of virus transmission. The ability to determine the effectiveness of measles elimination programs will also be enhanced by continued characterization of viruses from sporadic outbreaks of measles (1).

Virus isolation and genetic characterization can take several weeks to complete. Therefore, laboratory diagnosis of measles should always be based on detection of measles-specific IgM in serum. IgM enzyme immunoassay (EIA) can be completed in 1 day, and assay kits are available from several commercial sources. Specimens (urine, throat, or nasal) for virus isolation should be obtained at the same time that serum is drawn, since a delay in collection will reduce the chance of isolating the virus. However, urine or nasal specimens should not be substituted for serum specimens for measles diagnosis.

An Epstein-Barr virus-transformed, marmoset B lymphoblastoid cell line, B95a, is the preferred cell line for primary isolation of measles virus (5). These cells are as much as 10,000 times more sensitive for isolation of measles virus from clinical specimens than other commonly used cell lines, such as Vero and PMK. B95a cells are relatively easy to maintain in the laboratory, and the cytopathic effect (CPE) from measles infection is readily observed. However, laboratorians should note that this cell line does produce Epstein-Barr virus and should be handled as infectious material (Biosafety Level 2) at all times.

B95-8 cells are available from the American Type Culture Collection (# CRL 1612). When cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS), these cells will adhere to the surface of the culture vessel; the adherent cells are referred to as B95a. Cell growth is sustained in medium containing 8-10% FBS. FBS is used at a 2% concentration for cell maintenance during viral isolation. Optimal growth conditions for the cells are obtained by incubating the culture in a 5% CO_2 incubator at 37 C (See Note in section II). Cell stocks can be prepared by using standard cryoprotection medium (section III).

II. Material required

1. Dulbecco’s modified Eagle medium (DMEM):
   with 4,500 mg/L D-glucose (high glucose)
   with L-glutamine
   without sodium pyruvate
2. Antibiotics (100X)
   10,000 units/ml penicillin G
   10,000 ug/ml streptomycin sulfate
   in 0.85% saline
3. Trypsin-EDTA
   0.05% trypsin
   0.53 mM EDTA

in Hanks' balanced salt solution (HBSS) without Ca++ and Mg++

4. Fetal bovine serum (FBS)
5. 25-cm^2 culture vessels (T-25)
6. Inverted microscope, preferably with phase contrast optics
7. Cell culture incubator

Note: Minimum essential medium (MEM) may be substituted when CO_2 incubator is not available, however DMEM has been used with good results with the addition of 1% Hepes buffer and 1% $NaCO_3$ to improve buffering capacity.

*Use of trade names and commercial sources in this manual does not imply endorsement by the Centers for Disease Control and Prevention.

III. Cell Culture

Passage of B95a cell line

1. Perform all procedures under an appropriate biosafety cabinet. Because the B95a cell line contains potentially infectious Epstein-Barr virus, all discarded medium should be placed into a beaker containing 10% hypochlorite solution.

2. Allow DMEM and trypsin solution to warm to room temperature. It may be convenient to prepare small amounts of DMEM mixed with FBS.

3. Remove medium from T-25 flask and wash cells with either 3-5 ml of DMEM or rypsin by gently swirling the flask for about 10-20 seconds, then remove wash solution.

4. Add 1-2 ml of trypsin to the T-25 flask, screw the cap on tight, and rock the flask side to side. The cells will quickly become detached from the vessel surface in about 30-60 seconds. Dislodge the cells by rapping the flask with one hand. The cells should knock off easily.

5. Immediately add 5 ml of DMEM to the flask (total volume 7-6 ml) to collect the cells and transfer the fluid to a small tube to mix.
   The cells from one flask can be split 1:2, 1:3, or 1:4. Do not passage B95a cells at subcultivation ratios higher than 1:4. After 1:2 and 1:3 splits, the cells are usually ready to infect within 2 days.

6. Prepare the desired number of flasks, adding the appropriate volume of cell suspension to the flasks containing DMEM with10% FBS. Place the flasks in an incubator at 37 C. A CO_2 incubator is optimal for cell growth, but if one is not available, the substitution of minimum essential medium (MEM) for DMEM is recommended or DMEM with the addition of hepes buffer and sodium bicarbonate (NaCO3) may improve buffering capacity.

***Example*:** To set up 3 T-25 flasks from 1 T-25 flask, add 5 ml of DMEM to the trypsinized cells (total volume 8 ml). Vortex gently or pipet up and down. Distribute 2 ml (1:3 split) to each T-25 flask containing 10 ml of DMEM with 10% FBS. Cells should be ready to infect (75-85% confluent) in about 2-3 days.

Preparation of stock (frozen) B95a cells

It is extremely important to prepare multiple frozen stocks of B95a cells as soon as they are available in the laboratory. These cells will gradually lose their ability to form smooth, uniform monolayers, making detection of measles virus more difficult. It is recommended that B95a cells be passaged no more than 30-35 times.

Cells can be frozen using any standard cryopreservation technique. Commercial freezing medium is available or the reagents described below should be adequate.

Prepare a solution of 90% FBS and 10% DMSO (or glycerol) and store on ice. Make enough to allow for 1 ml for each vial of cells. For a 150-cm^2 flask, 10 vials of cells can be prepared. Decant the medium from a confluent 150-cm^2 flask of B95a cells, saving 10-20 ml of the medium in a centrifuge tube. Cells should be removed from the flask using a small amount of trypsin-EDTA. Add the cells to the decanted medium in the centrifuge tube and centrifuge the cell suspension at 400 x g for 5 minutes. Pour off the supernatant and add 10 ml of the required volume of cryoprotectant solution. Pipet gently up and down to mix and dispense 1 ml into each of 10 plastic cryovials. If available, the vials should be cooled using a programmed cell freezer or a commercial product designed for gradual temperature reduction. Store vials in gas-phase liquid nitrogen.

Shipment of growing B95a cultures

B95a cells can be transported at room temperature in a 25-cm^2 tissue culture flask. For shipment, flasks should be filled to the top with medium containing DMEM/2% FBS, and securely sealed. The package should include secondary watertight packaging required for infectious perishable material and have the appropriate labels on the outside. Additional information on shipping is given in section VII.

Upon receipt of shipped cells, examine the cell sheet. If many cells are free-floating, centrifugation (400 x g, 5 minutes) of the medium will recover the cells, which can be added back to the flask (or to another flask for passage). For a 25-cm^2 flask, add 10-15 ml of the medium back to the flask for cultivation.

IV. Processing of Specimens

As each specimen is logged in, a laboratory identification number and information for the patient and specimen should be recorded in the log book or spreadsheet. The specimen information may be helpful in identifying problems that may contribute to loss of virus and inability to make isolations. Problems in shipment or with the samples should be reported to the sender.

Important data to record:

Patient Information	Specimen Information
Age	Type (urine/throat swab/nasal washing/blood)
Date of birth	Date of collection of sample
Rash onset date	Volume (urine)
Blood draw date	Condition (temp. upon arrival)
IgM result	Actions taken (centrifugation, storage location)
Measles vaccination date	

Throat, nasal, or nasopharyngeal swabs or aspirates: If the specimen contains 2-3 ml of viral transport medium or phosphate-buffered saline (PBS), it can be either frozen at -70 C or stored at 4 C. If the original swab tube is sent, add 2 ml of DMEM, vortex to collect swab material, and drain the swab as much as possible against the side of the tube. If debris is heavy, centrifuge to remove.

Urine: If a centrifuge is available, transfer the urine to tube(s) to collect the sediment for 5-10 minutes at

400 x g. (A refrigerated centrifuge is recommended, but otherwise start with urine that has been chilled at 4 C). Resuspend sediment in 2 ml of cold DMEM. If a centrifuge is not available, transfer the urine to sterile, leak-proof containers and refrigerate.

Heparinized blood: Use a product designed to separate lymphocytes from peripheral blood during centrifugation.

Important: It is recommended that the sample be divided in case the sample is determined to contain bacteria or other contaminants or toxic components. Do not routinely filter clinical specimens before inoculation. However, if a culture is found to be contaminated on the first isolation attempt, the remaining specimen can be filtered. To filter the specimen, bring the volume to 1-2 ml with DMEM, then filter contents through a syringe filter (0.45 um) and collect in a sterile tube.

V. Inoculation of Specimens for Measles Isolation

1. Cells should be at 75-85% confluency when specimens are inoculated.

2. For initial infection of B95a cells (T-25 flask), decant medium and add specimen (in total volume 1.5 ml, sufficient to keep monolayer from drying out). Clinical specimens usually consist of urine sediment, throat swab fluid, or centrifuged nasal specimens that have been resuspended in a small volume(1.0 - 2.0 ml) of tissue culture medium. See section IV regarding the processing of specimens.

3. Incubate at 37 C for 1 hour to allow the virus to adsorb. After the adsorption period, observe the cells under the microscope to determine whether the sample was toxic to the cells (rounding of cells, cells floating). The cells will usually recover but may need a change of medium. This is virus passage #1.

4. Add 10 ml of DMEM containing 2% FBS, with antibiotics.
   The following day, check the cells. Tiny holes often can be seen as early as 1 day after infection by observing the cell layer against a light source.
   If holes in the monolayer are observed, examine by microscope to see if fused cells (syncytia) are visible. Sometimes infected foci detach from the monolayer and float into the medium. Be aware that some holes in the monolayer may appear which are not caused by measles. Examine the cell sheet for signs of bacterial contamination. Discard the flask if yeast or bacteria is present.

5. Treatment of specimens with antibiotics: At the time of inoculation, penicillin/streptomycin may be added to the media. When supplied as 10,000 units penicillin and 10,000 ug per ml streptomycin, add at 1:50-1:100 so the final concentration in the medium will be 100-200 units penicillin and 100-200 ug/ml streptomycin.

6. Change the cell medium if it appears too acidic (orange-yellow). Centrifuge to collect cells if they have detached from the cell sheet.

7. Passage cells when monolayer is confluent, usually 2 days after inoculation. Transfer medium to a tube and add the trypsinized cells, and centrifuge (400 x g for 5-10 minutes). Resuspend cells in a small amount of medium (6 ml) and then gently mix or vortex. Following the initial infection, it is recommended to use all of the cells for the next cell passage, distributing half of the cell mixture to each of 2 T-25 flasks (virus passage #2). Add DMEM with 2% FBS.

8. Check the flasks daily. It may be necessary to split the cells again within 1-2 days. For passage #3, use a 1:2 split and discard the remaining cells. If no CPE is observed after cultivation of the passage #3 cells, then discard.

9. When measles CPE is observed, continue to grow the cells (change the medium, if necessary) until the CPE becomes extensive. It may be necessary to re-distribute/passage the cells 1-2 times to allow the infection to spread before cells become overgrown. When CPE has spread to at least 50-75% of the cell layer, the virus has reached a suitable titer for viral stock.

10. To prepare a viral stock, scrape the cells into the medium with a cell scraper. Mix and distribute to 4-6 vials and store at -70 C.

If typical measles CPE is observed, confirmation of measles virus should not be necessary if the clinical symptoms were consistent for a measles case.

VI. Immunofluirescence

The immunofluorescence assay (IFA) described here uses a monoclonal antibody to detect the nucleoprotein of measles virus in infected cells. The infected cells are fixed onto a microscope slide. Binding of the measles-specific antibody is detected by using a goat anti-mouse antibody that is conjugated to fluorescein isothiocyanate (FITC). Binding of the detector antibody is visualized by fluorescence microscopy.

Both direct and indirect IFA kits are commercially available from a number of sources. This discussion will describe the Light Diagnostics Measles Indirect Immunofluorescence Assay from Chemicon, Inc. (catalog #3187).

It is also possible to configure an indirect IFA without using a commercial kit. Most monoclonal antibodies to the nucleoprotein will perform well in the IFA procedure described below. In particular, Mab:KK2 is available from the WHO measles reagent bank maintained by Dr. Fabian Wild in Lyon, France. Monoclonal antibodies directed against other viral proteins, such as the hemagglutinin and fusion proteins, may recognize conformational epitopes that are not stable after acetone fixation.

Chemicon Procedure

1. Place 1 ml of infected cell suspension (of total 10 ml cell suspension from a T-25 flask) into a small centrifuge tube and pellet the cells by centrifugation at 400 x g for 10 minutes at 4 C. Decant the supernatant medium and resuspend the cells in 1.0 ml of PBS. Spread about 15 ul into one chamber of a microscope slide, using a micropipet or Pasteur pipette and allow the cells to air dry on the slide. Remember to include the uninfected cell controls.

2. After the cells are completely air-dried, place the slide in a staining jar containing ice-cold 80% acetone (in water) for 10 minutes. Remove the slide and allow it to air dry.

3. Prepare the PBS-Tween buffer supplied with the kit (PBS, 0.1% Tween 20).

4. Overlay the cell spots on the slides with one drop of the measles monoclonal antibody.

5. Incubate the slide at 37 C for 30 minutes to 1 hour in a humid chamber. Petri plates containing one wet paper towel will work very well.

6. Wash the slides for 15-20 seconds in the PBS-Tween buffer and shake off excess buffer.

7. Add one drop of anti-mouse IgG/FITC conjugate to the cell spot.

8. Incubate the slide at 37 C for 30 minutes in a humid chamber.

9. Wash the slides for 15-20 seconds in the PBS-Tween buffer and shake off excess buffer. Prepare

slides for viewing with mounting fluid and a cover slip.

10. Observe for fluorescence by using a fluorescence microscope. FITC absorbs at 495 nm with peak emission at 525 nm. Under these conditions, positively stained cells will show a granular, green fluorescence in the cytoplasm. The Evan's blue counterstain will appear red.

VII. Collection and Shipping of Clinical Specimens

Specimens for virus isolation should be obtained as soon as possible when measles infection is suspected, preferably at the onset of rash when the serum sample is collected. Urine and respiratory samples are both good clinical specimens for viral isolation. With very young patients, a respiratory sample (throat swab) may be easier to obtain than urine. Other types of respiratory samples may be more readily obtained in a clinic or hospital where the equipment is available and it is recommended to take advantage of the opportunity. Protocols are described below.

A. Respiratory specimens

Materials:
1. Sterile swabs
2. Sterile saline
3. 3-ml aliquots of viral transport medium (VTM; sterile PBS or suitable isotonic solution such as HBSS, containing antibiotics:100 units/ml penicillin, 100 ug/ml streptomycin) and either 2% fetal bovine serum or 0.5% gelatin in 15 ml polycarbonate or polystyrene centrifuge tubes
4. 5-ml plastic syringes
5. Plastic aspirators or 30-ml syringe
6. Cryovials
7. Styrofoam shipping containers

Instructions:

Attempt to obtain the sample as soon as possible after onset of rash. Virus is most frequently recovered within the first 3 days following rash (but up to 7 days after rash onset is acceptable).

A nasal wash (nasopharyngeal aspirate) can be obtained by using a syringe attached to a small piece of plastic tubing. After placing about 3-5 ml of saline in the nose, aspirate as much of the material as possible and add to the centrifuge tube containing the VTM. (In a clinic or hospital setting, if available, a vacuum may increase the recovery of fluid.) Rinse the syringe and collection tubing into the VTM.

Alternatively, sterile swabs can be used to obtain throat and naspharyngeal specimens. A throat swab is taken by rubbing the posterior nasal passages with a dry sterile cotton swab. Place swab in a tube containing 2-3 ml of VTM. The swab can be broken off into the tube of VTM.

Place respiratory specimens at 4 C and ship to an appropriate laboratory with cold packs. Refrigeration of samples is adequate if cold shipment can be arranged within about 48 hours. If there is a delay, and it is possible, freeze the samples at -40 to -70 C and ship frozen on dry ice.

B. Urine specimens

Materials:
1. Urine collection cups, preferably with lid.
2. 50-ml polystyrene screw-cap centrifuge tubes.
3. PBS or DMEM
4. Cryovials

5. Shipping containers

Urine should be collected within 7 days of rash onset (within 1-3 days if possible). First- morning voided specimens are ideal, but any urine collection is adequate. Collect 10-50 ml of urine in a urine specimen container.
Centrifuge the urine specimen as soon as possible after collection. After collection, keep the specimen cold (refrigerator or wet ice). Transfer the urine specimen to a 50-ml plastic conical centrifuge tube and centrifuge at 400 x g for 5-10 minutes at 4 C to collect the sediment. Resuspend the sediment in 2-3 ml of VTM (above) or any cell culture medium (DMEM, EMEM, RPMI plus antibiotics). Preferably, specimens that have been centrifuged and resuspended should be frozen at -70 C and shipped on dry ice. If dry ice is not available, however, they can be stored at 4 C and shipped on wet ice or cold packs.
If centrifugation is not available, do not freeze the urine sample. The entire urine specimen should be stored at 4 C, and shipped to the lab on wet ice. It is best to have the specimen shipped to a viral laboratory within 48 hours so that it can be processed and frozen at -70 C for optimal virus recovery. Seal the specimen container tightly to prevent leakage.

C. Blood samples

Virus can also be isolated from lymphocytes. If it is possible to collect several milliliters of heparinized blood, the lymphocytes will be a good source of virus. The whole blood should be stored at 4 C and transported to the laboratory within 48 hours of collection.

D. Shipping of clinical specimens and viral isolates

It is recommended to use containers made specifically for shipping infectious substances, for example, the Saf-T-Pak system.

For shipping of viral isolates in cell culture, it is best to use a plastic 25-cm^2 tissue culture flask. Cells should be infected 1-2 days before shipping. Before shipment, fill the vessel to the top with DMEM (plus antibiotics and 2% FBS). Screw the top on tightly and seal with plastic film or tape. Place the flask in a leak-proof container, such as a zip-lock plastic bag with absorbent material, and ship at room temperature.

Infected cells can be pelleted, resuspended in a small volume of DMEM, and frozen at -70 C before shipping on dry ice.

E. Shipping information

CDC is a designated WHO measles strain bank and will accept viral isolates or clinical specimens from cases of measles for genetic characterization.

It is important to notify the Measles Virus Section before shipping, particularly for international shipments.

Tel: 404-639-1156 -3512
FAX: 404-639-4187
E-mail: jrota@cdc.gov

Specific instructions for shipping will be provided. Viral isolates and clinical specimens arriving from outside of the USA will require a CDC import permit, available upon request (a faxed copy is acceptable).

For international shipments, a copy of the import permit must accompany the shipping documents. A copy of the label must be affixed to the outer shipping container.
Please call, fax or e-mail to obtain a valid permit and shipping label if the date has expired or if the permit has been lost.

Ship to:

Dr. William J. Bellini
Measles Virus Section, C-22
DASH Group #81
Centers for Disease Control and Prevention
1600 Clifton Road
Atlanta, GA 30333 USA Tel: 404-639-3512

VIII. REFERENCES

1. Rota J.S., Rota P.A., Redd S.B., Redd S.C., Pattamadilok S., and Bellini W.J. Genetic analysis of measles viruses isolated in the United States 1995-1996. Journal of Infectious Diseases 1998; 177:204-8.

2. Rota J.S., Heath J.L., Rota P.A., King G.E., Celma M.L., Carabaña J., Fernandez-Muñoz R., Brown D., Jin L., and Bellini W.J. Molecular epidemiology of measles virus: Identification of pathways of transmission and the implications for measles elimination. Journal of Infectious Diseases 1996; 173:32-7.

3. Rota P.A., Rota J.S., and Bellini W.J. Molecular epidemiology of measles virus. Seminars in Virology 1995; 6:379-86.

4. World Health Organization. Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. Weekly Epidemiological Record 1998; 73:265-72.

5. Kobune F., Sakata H., and Sugiura A. Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. Journal of Virology 1990; 4:700-5.